Final Technical Report (0003AB): A Novel 3 Dimensional Biomaterial for the Production of Multiple Blood Cell Types

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Support of wounded personnel is intimately tied to the availability of sufficient quantities of effective blood cells, including conventional red blood cells and platelets and novel therapeutic T lymphocytes and white blood cells. Phase I demonstrated the feasibility of utilizing a novel, biocompatible 3-D device, termed Cellfoam, to support the *in vitro* production of red blood cells, white blood cells and platelets from human hematopoietic progenitor cells (HPCs). Previous work has shown that Cellfoam enables the long-term, cytokine-free culturing of HPCs while maintaining their multipotency and longevity. In Phase I, HPCs cultured in the presence of selected cytokines were evaluated for cell expansion, phenotype, multipotency and lineage progression over 4 weeks. These studies demonstrated the ability to produce maturing blood progenitor cells from HPCs cultured in Cellfoam. Such culturing enabled cell expansion of up to 100-fold and the acquisition of phenotypic traits consistent with lineage-targeted differentiation. Importantly, cytokine-supplemented HPCs cultures in Cellfoam exhibited enhanced maintenance of immature progenitors relative to controls, corroborating data that Cellfoam offers an enabling environment which selectively preserves immature, multipotent progenitors. Building on this encouraging work, Phase II will develop Cellfoam prototype systems for the first-ever sustained, high-capacity production of functional blood cells from stable HPCs.

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Introduction:

This final technical report presents the results of Cytomatrix's Phase I SBIR project (contract number DAAHO1-97-C-R121) entitled "A novel 3 dimensional biomaterial for the production of multiple blood cell types." The Phase I project examined the feasibility of utilizing a novel, biocompatible, porous three dimensional device, termed Cellfoam, to support the *in vitro* production of red blood cells, white blood cells and platelets from primitive human $CD34^+$ hematopoietic progenitor cells (HPCs). Preliminary work conducted prior to the Phase I contract had shown that Cellfoam devices are capable of maintaining immature HPCs in the absence of co-cultured stromal cells or supplemented cytokines, and that $CD34^+$ HPCs in short-term Cellfoam cultures retain the multipotent ability to produce myeloid, lymphoid and erythroid progeny. Phase I demonstrated that Cellfoam is able to support the survival of both immature and lineage-committed HPCs and to facilitate their targeted differentiation in the presence of selected exogenous cytokines into erythroid, myeloid and platelet progeny. Given the limitations of current-day sources of blood and blood components, the development of a safe and readily available alternative blood source would have great impact on both civilian and military populations. Of particular relevance to the military, such a system may address the large unpredictable demand for blood products needed to treat personnel injured in combat or exposed to biological and/or chemical agents during warfare. Immediate treatment and ongoing support of wounded or exposed personnel are intimately tied to the availability of sufficient quantities of effective blood products.

Phase I has clearly demonstrated the ability to produce maturing blood progenitor cells from hematopoietic stem cells (HSCs) cultured in Cellfoam. These findings, coupled with Cellfoam's powerful ability to support long-term HSC survival and expansion in the absence of cytokines while preserving multipotency, provide the foundation for developing an advanced system for the extended preservation of pluripotent HSCs and the long-term production of differentiated blood cells. HSCs are long-lived, self-renewing cells that give rise to all lineages of mature, differentiated blood cells, namely cells of the lymphoid, myeloid and erythroid lines. As such, HSCs are prime candidates for use in *in vitro* systems as precursor cells capable of generating the complete and diverse cellular constitution of whole blood. The overall goal of this research is to develop Cellfoam devices capable of sustaining primitive, pluripotent progenitor cells and their renewable differentiation into specific blood cell lineages, including white blood cells, T cells, NK cells, CTLs, red blood cells and platelets. Specifically, Phase I has employed Cellfoam to produce particular cytokine-targeted hematopoietic lineages. Building on this work, Phase II will develop a high-capacity Cellfoam prototype system for the sustained production of functional blood cell progeny from a stable population of self-renewing HPCs.

Operating from the basis that the 3 dimensional Cellfoam environment enhances the survival and subsequent differentiation of primitive progenitor cells into mature red blood cells, white blood cells, and platelets, relative to conventional control systems, the specific objectives of Phase I were:

- **Task 1. To determine the ability of HPCs and erythrocyte progenitor cells (EPCs) cultured in Cellfoam to generate erythroid progeny *in vitro*.** Studies are underway to determine the efficacy of using immature HPCs ($CD34^+CD38^-$) and committed erythroid progenitors ($CD34^+CD71^+$) to generate red blood cell progeny *in vitro*.

- **Task 2. To determine the ability of HPCs and myeloid progenitor cells (MPCs) cultured in Cellfoam to generate myeloid progeny *in vitro*.** These studies examine the efficacy of using immature HPCs ($CD34^+CD38^-$) and committed myeloid progenitors ($CD34^+CD33^+$) to generate mature granulocytes and monocytes *in vitro*.

- **Task 3. To determine the ability of HPCs and platelet progenitor cells (PPCs) cultured in Cellfoam to generate megakaryocytes and platelet progeny *in vitro*.** Immature HPCs ($CD34^+CD38^-$) and committed thrombocyte progenitors ($CD34^+CD61^+$) are studied for the ability to generate megakaryocytes and mature platelets *in vitro*.

Framework:

The goal of Phase I was to evaluate the efficiency with which and duration for which immature HPCs ($CD34^+CD38^-$) and committed progenitors ($CD34^+lin^+$) cultured in Cellfoam units are able produce maturing blood cells from different source cells. The general experimental framework used throughout the DARPA studies entailed the culture of $CD34^+CD38^-lin^-$ (immature HPCs), $CD34^+CD38^+lin^-$ (intermediate HPCs), and $CD34^+CD38^+lin^+$ (committed progenitors) derived from human bone marrow (Poietic Technologies, MD) which had been isolated by flow cytometry-based fluorochrome-tagged antibody cell sorting. Additionally, $CD34^+$ cells purified by immunomagnetic beads (Dynal) were added to the experimental design as an additional control to evaluate the performance of unsorted HPCs. Cells from these populations were inoculated into Cellfoam or parallel liquid culture controls in fibronectin-coated plastic wells and cultured under conditions defined to support *in vitro* differentiation of red cell progenitors [Task I; RPMI +10%FCS, IL-3(50ng/ml), SCF(50ng/ml) and erythropoietin (3U/ml)], white blood cells [Task II, RPMI +10%FCS, IL-3(50ng/ml), SCF(50ng/ml) and GMCSF (20ng/ml)], and megakaryocytes [Task III; RPMI +10%FCS, IL-3(50ng/ml), SCF(50ng/ml) and thrombopoietin (10 ng/ml)], for 14-21 days. Human bone marrow was chosen as the starting material because human bone marrow samples became commercially available after the submission of the original proposal. The availability of this source obviates the need to pool and cryopreserve variable cord blood samples, thus eliminating extensive manipulations and reducing confounding variables in these procedures.

In all experiments, Cellfoam units were coated with fibronectin to assist in providing adhesion sites for HPCs and to create a parallel environment for control fibronectin-coated plastic wells. Each human bone marrow sample was used fresh. The mononuclear fraction of the bone marrow samples was first separated by ficoll gradient purification and treated with 0.15M ammonium chloride to lyse the red blood cells; the remaining nucleated cells were stained with fluorescently labeled antibodies to CD34, CD38 and lineage markers (Becton Dickinson).

For analysis of erythropoiesis, mononuclear cells were sorted by flow cytometry into $CD34^+38^-71^-$ (immature HPCs); $CD34^+38^+71^-$ (intermediate HPCs); and $CD34^+38^+71^+$ (committed erythroid progenitors) cells. Thrombopoiesis studies utilized $CD34^+38^-61^-$ (immature HPCs); $CD34^+38^+61^-$ (intermediate HPCs); and $CD34^+38^+61^+$ (committed platelet progenitors) cells. Finally, myelopoiesis trials used $CD34^+38^-33^-$ (immature HPCs); $CD34^+38^+33^-$ (intermediate HPCs); and $CD34^+38^+33^+$ (committed white cell progenitors) cells. Subsequent to FACS sorting, 5×10^4 to 1×10^5 cells from each sorted sub-population (i.e. $CD34^+38^-lin^-$, $CD34^+38^+lin^-$, and $CD34^+38^+lin^+$) cells were cultured in Cellfoam and plastic control cultures (plastic multi-well plates coated with fibronectin) under conditions defined to support the *in vitro* formation of differentiating progeny as outlined above. Media was changed twice weekly. At respective time-points, cells were harvested from the Cellfoam cultures and plastic controls. Total cell count was established using a hemacytometer. Cell morphology was examined by light microscopy to evaluate morphology. Phenotype was evaluated by flow cytometry analysis. Harvested cells were stained with antibodies to CD45 (to gate on hematopoietic cells v. stromal cells), CD34, CD38 and CD71, CD61 or CD33. Using three color FACS analysis, the presence of immature $CD34^+$ cells, committed hematopoietic progenitors ($CD34^+CD38^+lin^+$), as well as more mature ($CD34^-CD38^-CD71^-$) cells was determined. Studies included appropriate FACS controls, including matched isotype antibodies to establish positive and negative quadrants, and single color stains to establish compensation. At least 10,000 list mode events were collected.

Findings:

The tasks outlined in the Phase I proposal were intended to demonstrate the feasibility of producing maturing blood cells in Cellfoam. Because the three tasks embodied similar lines of investigation differing predominantly in the cell subtype being analyzed (i.e. red cells, white cells or platelets), this review considers the data across the tasks and will evaluate the following data:

- overall cell expansion
- $CD34^+$ progenitor maintenance
- lineage targeted facilitation
- phenotype evaluation

To evaluate the culture and differentiation relative to existing data, the scope of the Phase I work was extended to include not only sorted cells isolated into specific maturation groups (herein designated $CD34^+38^-lin^-$, $CD34^+38^-lin^-$ and $CD34^+38^+lin^+$) but also bulk purified 34^+ bead purified cells. This enabled resolution of potential advantages and disadvantages of purified cell subpopulations relative to bulk bead separated cells. Additionally, the time intervals examined were extended to four weeks because initial experiments indicated that useful information would be obtained at later times.

Cell Expansion:

An important aspect of the Phase I work was to evaluate the overall ability of the Cellfoam system to produce expanded numbers of differentiating progeny. Relative expansion is an important attribute of any scaled-up system aiming to produce enough cells to be of clinical value. A proven approach to increasing cell numbers while fostering their differentiation is the augmentation of stem cell cultures with particular sets of cytokines. Much work has been done to show that specific cytokines can induce the lineage targeted differentiation of stem cells toward particular lineages. With this in mind, each task examined the expansion of cell numbers in Cellfoam and plastic-fibronectin controls when supplemented with cytokines aimed at stimulating the production of red cell (IL-3(50ng/ml), SCF(50ng/ml) and erythropoietin (3U/ml)], white blood cell [IL-3(50ng/ml), SCF(50ng/ml) and GMCSF (20ng/ml)], and megakaryocyte [IL-3(50ng/ml), SCF(50ng/ml) and thrombopoietin (10 ng/ml)] progeny.

Erythropoietin cultures: In erythropoietin (Epo)-supplemented cultures, both plastic and Cellfoam were observed to support expansion of total cell numbers. As shown in Figure 1, overall cell numbers in Cellfoam were slightly lower than, yet not outside the range of statistical variation of, parallel plastic controls. Within either plastic or Cellfoam, initial (2 week) expansion was similar for all sorted subsets. After two weeks in both plastic and Cellfoam cultures, $CD34^+$ bead purified cells led to similar expansion as $CD34^+CD38^+71^+$ sorted cells; both of these sets outperformed $CD34^+CD38^+71^-$ and $CD34^+CD38^-71^-$ sorted cells. The performance of the $CD34^+CD38^+71^+$ sorted cells may reflect their maturity and their associated responsiveness to cytokines. While the bead purified cells continued to expand in both Cellfoam and plastic, the $CD34^+CD38^+71^+$ cells held steady in number in Cellfoam and exhibited a decline in numbers in plastic-Epo cultures from 2-4 weeks. The $CD34^+CD38^+71^-$ population in both Cellfoam and plastic exhibited an initial increase in cell number at 2 weeks followed by a decrease at 3 and 4 weeks. The $CD34^+CD38^-lin^-$ set in both Cellfoam and plastic exhibited a steady decline in numbers. Expressed in terms of average fold expansion, bead purified cells consistently showed the highest levels of expansion (Table I). In both Cellfoam and plastic cultures, the presence of differentiating progenitors was detected through four weeks in the presence of cytokines. This was an unexpected and encouraging finding as the addition of these high levels of cytokines was originally envisioned to deplete cell content by 3 to 4 weeks. These data indicate the possibility of achieving sustained differentiation of progenitors.

Figure 1. Total cell numbers in erythropoietin-supplemented Cellfoam and plastic cultures

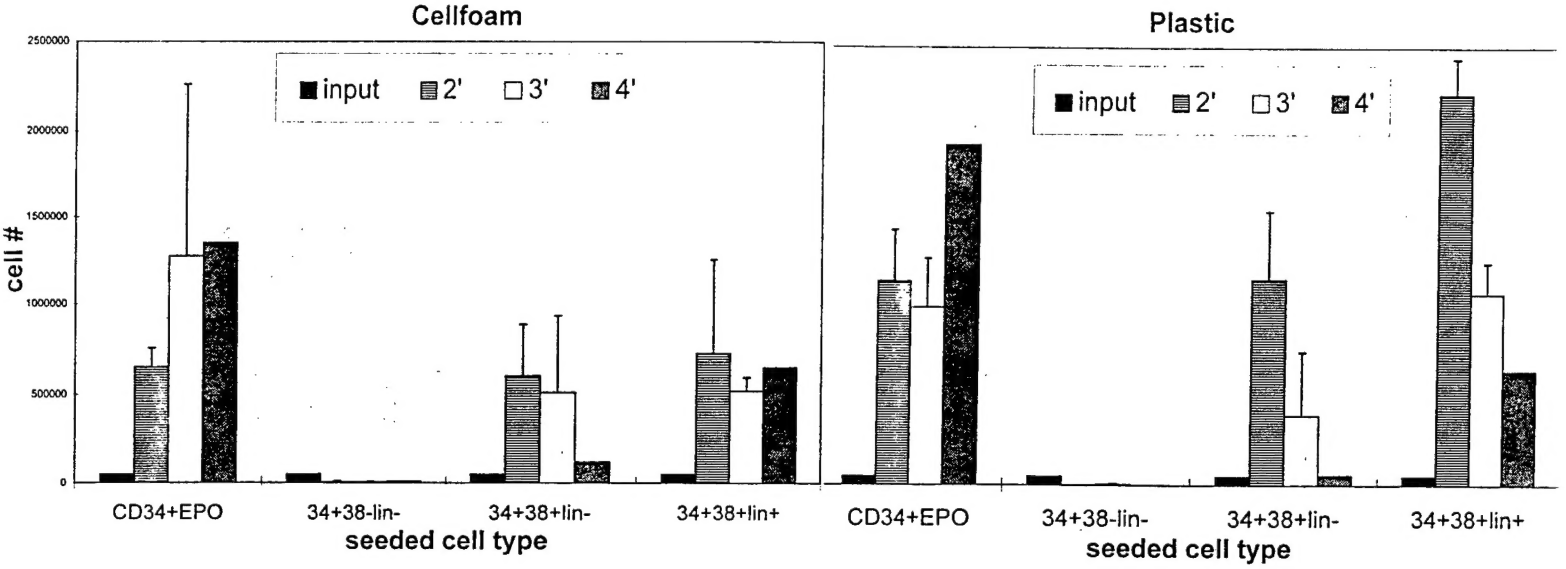


Table I. Average fold expansion in erythropoietin-supplemented Cellfoam and plastic cultures

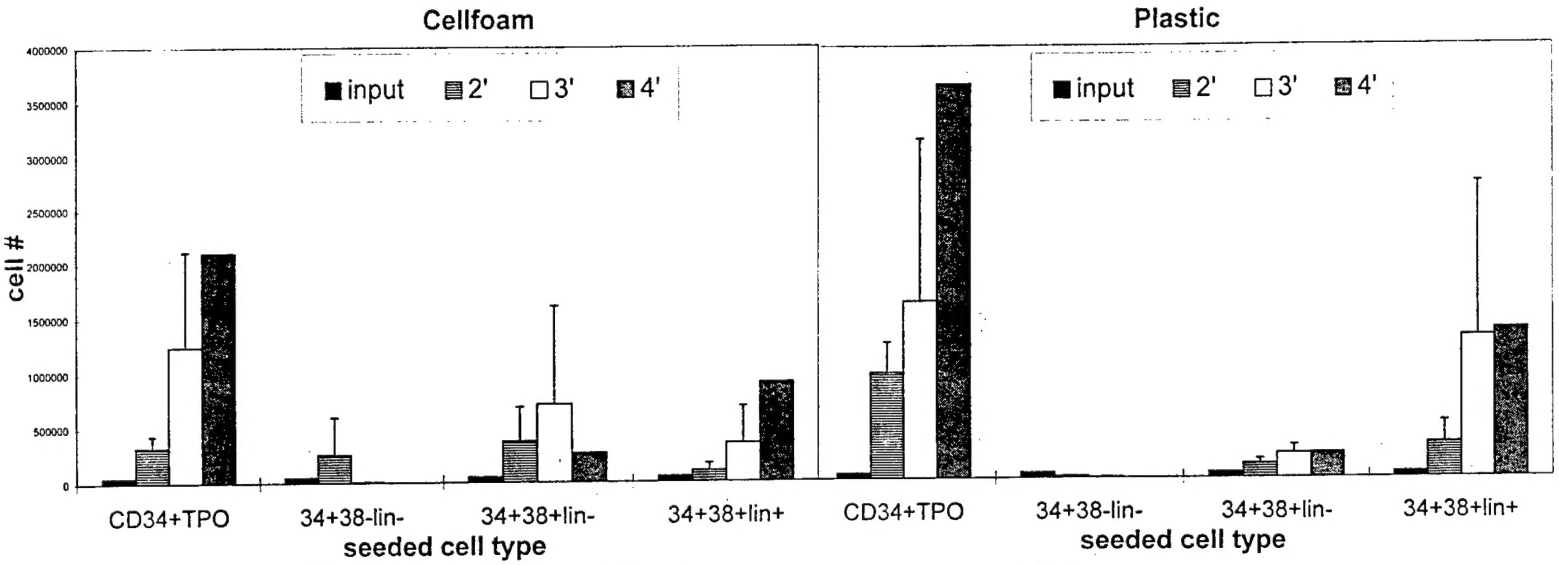
	2 wks		3 wks		4 wks	
	Cellfoam	plastic	Cellfoam	plastic	Cellfoam	plastic
bead $CD34^+$	13	22	25	20	27	39
$CD34^+CD38^+71^-$	12	16	10	8	2	1
$CD34^+CD38^+71^+$	15	17	10	21	13	13

Thrombopoietin cultures: The findings described above for Epo-supplemented cultures bear similarity to the thrombopoietin (Tpo)-supplemented cultures. Consideration of the fold expansion again highlights the considerably greater expansion potential of bead purified $CD34^+$ cells using Tpo (Table II). In general, expansion in Tpo-supplemented plastic cultures was slightly, although not significantly, higher than in Cellfoam. Bead purified cells exhibited a greater capability for expansion than $CD34^+CD38^+61^+$ cells, which outperformed $CD34^+CD38^+61^-$ cells (Figure 2). $CD34^+CD38^-lin^-$ cells again revealed little capacity for expansion; however, at 2 weeks the number of cells from this subset did increase in Cellfoam. In both bead purified and $CD34^+CD38^+61^+$ cultures, the number of cells increased from 2 to 3 to 4 weeks. This reinforces the possibility of achieving extended differentiation, as raised in the Epo experiments. The continued increase in cell number in $CD34^+CD38^+61^+$ sorted cells in Cellfoam at 4 weeks suggests that long lived cells capable of extended proliferation may be present within this subset and sustained to a greater extent in Cellfoam. The steadily increasing and prolonged nature of this cell expansion in Cellfoam versus plastic is consistent with more regulated cell division in Cellfoam.

Table II. Average cell expansion in thrombopoietin-supplemented Cellfoam and plastic cultures

	2 wks		3 wks		4 wks	
	Cellfoam	plastic	Cellfoam	plastic	Cellfoam	plastic
bead $CD34^+$	7	16	25	33	42	73
$CD34^+CD38^+61^-$	8	2	14	5	5	5
$CD34^+CD38^+61^+$	2	6	8	27	18	27

Figure 2. Total cell numbers in thrombopoietin-supplemented Cellfoam and plastic cultures



GMCSF cultures: The general pattern of bead purified cells leading to the highest overall expansion which emerged for Epo- and Tpo- supplemented Cellfoam and plastic cultures was found to pertain to GMCSF-augmented cultures as well (Figure 3). $CD34^+CD38^-lin^-$ cells again performed poorly. Conversely, as opposed to Epo and Tpo cultures where the $CD34^+CD38^+lin^-$ cells did not perform as well as the $CD34^+CD38^+lin^+$ cells, $CD34^+CD38^+33^-$ cells in GMCSF cultures led to expansion rates similar to, and in some instances superior to, $CD34^+CD38^+33^+$ cells raising the possibility that this subset may have sustained proliferative capacity. Cell expansion was again observed to rise (bead purified cells), remain unchanged ($CD34^+CD38^+33^+$ cells) or decline only slightly ($CD34^+CD38^+33^-$ cells) through the 4 week time-point, again a sign of potential longevity of the system. Total cell expansion using GMCSF was lower than using Epo or Tpo (Table III).

Figure 3. Total cell numbers in GMCSF-supplemented Cellfoam and plastic cultures

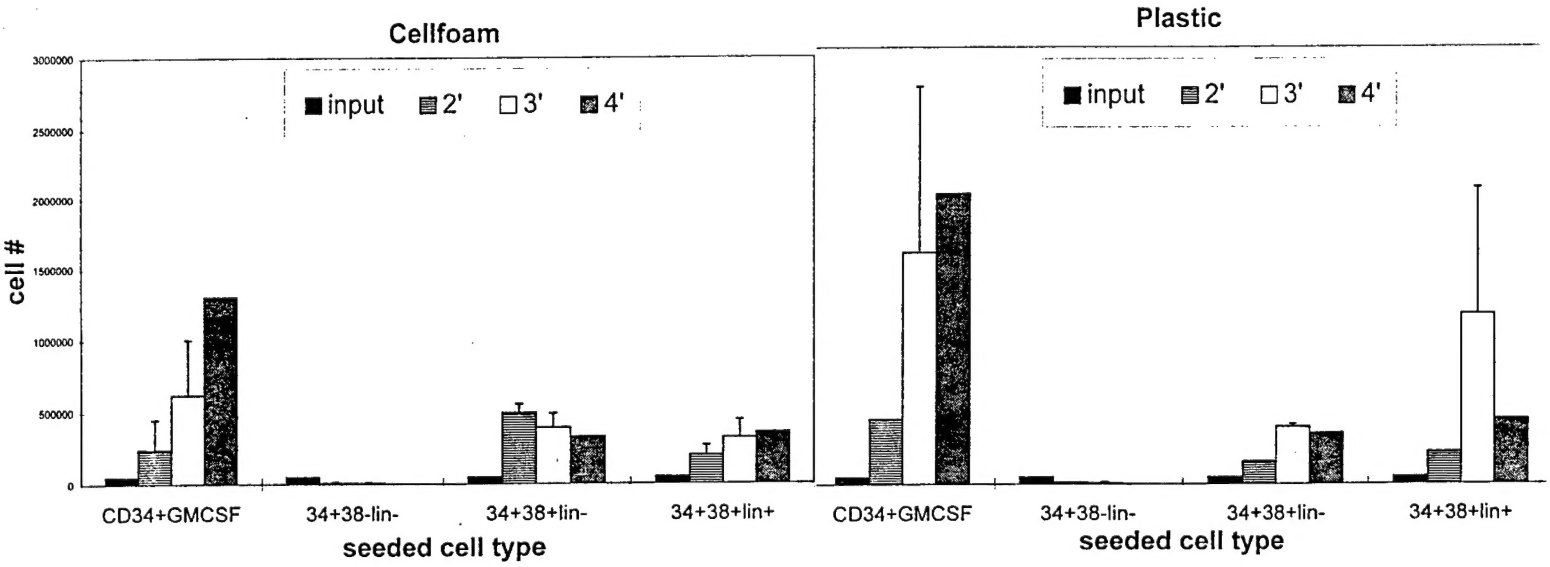


Table III. Average cell expansion in GMCSF-supplemented Cellfoam and plastic cultures

	2 wks		3 wks		4 wks	
	Cellfoam	plastic	Cellfoam	plastic	Cellfoam	plastic
bead $CD34^+$	5	7	12	2	26	40
$CD34^+CD38^+33^-$	10	4	9	8	7	7
$CD34^+CD38^+33^+$	4	6	6	22	7	9

Expansion conclusions: Augmentation of Cellfoam and plastic progenitor cell cultures with lineage-inducing cytokines (erythropoietin, thrombopoietin, or GMCSF) in conjunction with IL-3 and SCF leads to measurable expansion of bead purified $CD34^+$ and $CD34^+CD38^+lin^+$ sorted cells. This is consistent with the fact that bead-purified populations contain $CD34^+CD38^+lin^+$ subsets, as well as $CD34^+CD38^+lin^-$ and $CD34^+CD38^-lin^-$, all of which may provide different, perhaps synergistic capabilities in stem cell maintenance or differentiation. Intermediate progeny ($CD34^+CD38^-lin^-$) are depleted over time upon cytokine augmentation with little apparent capacity to expand using the chosen cytokines. Expanded cell numbers were achieved at the latest time-point examined (4 weeks), particularly with bead separated $CD34^+$ cells in plastic and Cellfoam and with $CD34^+CD38^+lin^+$ sorted cells in Cellfoam where overall cell numbers continued to rise. The sustained expansion of $CD34^+CD38^+lin^+$ cells (which are relatively mature) is encouraging as they are the direct source of mature cells. The observation that Cellfoam appears to foster longer-term function of this population is consistent with the well regulated culturing of progenitors in the 3 dimensional Cellfoam environment.

Together, these observations help to define the structure and design of an advanced Cellfoam blood cell production system capable of the long-term preservation of HSCs while achieving the simultaneous elaboration of mature progeny. The ability of immature progenitors capable of long-term survival and progeny formation to survive in cytokine-augmented cultures provides a basis for a multi-compartment stem cell preservation and differentiation system in which compartments for blood cell differentiation may be joined to, and supplied from, separate, cytokine-free compartments for stem cell preservation and expansion. The integration of the separate chambers may be mediated by a perfusion apparatus, which may not only serve as the vehicle for transferring cells from one compartment to another, but may also impart benefits to stem cell survival in and of itself. This approach is discussed in more detail toward the end of the report where the fabrication of a Cellfoam perfusion prototype is presented.

CD34 Positivity:

While overall cell number is important for scaled-up blood cell production systems, a critical component of long-lived systems is the ability to maintain immature progenitors during culture. Cytomatrix has already demonstrated that Cellfoam preserves and expands HSC content and functionality in long-term (8-10 wk+) cytokine-free cultures, a capability not demonstrated by other systems. An important issue, then, becomes the extent to which progenitors (defined as expressing CD34 surface antigen) are maintained in cultures supplemented with high levels of exogenous cytokines designed to promote lineage-specific differentiation. While the high levels of cytokines used in the current experiments were likely to drive progenitors into proliferation and differentiation pathways, making the status of the $CD34^+$ compartment uncertain, the continued presence of these cells would serve as an indication of the long-term capabilities of the approach. An important driver in the development of sustained blood cell production systems must entail acknowledgment of the preservation of the most immature and long-lived progenitors. To examine the survival of such cells in cytokine-supplemented cultures, cell populations isolated from Cellfoam and plastic cultures were analyzed by flow cytometry for the continued presence of immature cells bearing the CD34 antigen, a general marker for overall stem cell primitiveness.

Erythropoietin cultures: The exposure of HSCs to cytokines has been shown to deplete the content of this pool of cells over time. Consistent with this principle, all categories of cells (bead purified, $CD34^+CD38^-71^-$, $CD34^+CD38^+71^-$ and $CD34^+CD38^+71^+$) exhibited a steady decline in $CD34^+$ content over the 4 week culture period in which cells were exposed to IL-3, SCF and erythropoietin (Figure 4). The percent $CD34^+$ was similar at 2 and 3 weeks in Cellfoam and plastic. At 4 weeks, the percent $CD34^+$ was greater in Cellfoam than in plastic, especially in the bead purified cell subset (24% in Cellfoam versus 15% in plastic, representing at 62.5% increase in $CD34^+$ content). Importantly, the primary flow cytometry data revealed that a population of $CD34^+38^-$ cells was maintained in Cellfoam (Figure 5). The presence of this somewhat more stable pool of immature precursors may be one reason that Cellfoam was able to sustain ongoing cell number increases at times (especially 4 weeks) when plastic cultures had begun to drop off. This would again be consistent with Cellfoam's prevailing effect of appropriate regulation of progenitor cells. Interestingly, the $CD34^+CD38^+71^-$ cells yielded a higher percentage of $CD34^+$ cells than the $CD34^+CD38^+71^+$ cells or bead purified cells at 4 weeks. One potential explanation for this is that these cells overall were less responsive to cytokines and that the more quiescent Cellfoam environment favors $CD34^+$ maintenance via appropriate cell regulation.

Figure 4. Percent CD34+ cells in erythropoietin-supplemented Cellfoam and plastic cultures. +-- = $CD34^+38^-71^-$; ++- = $CD34^+38^+71^-$; +++ = $CD34^+38^+71^+$

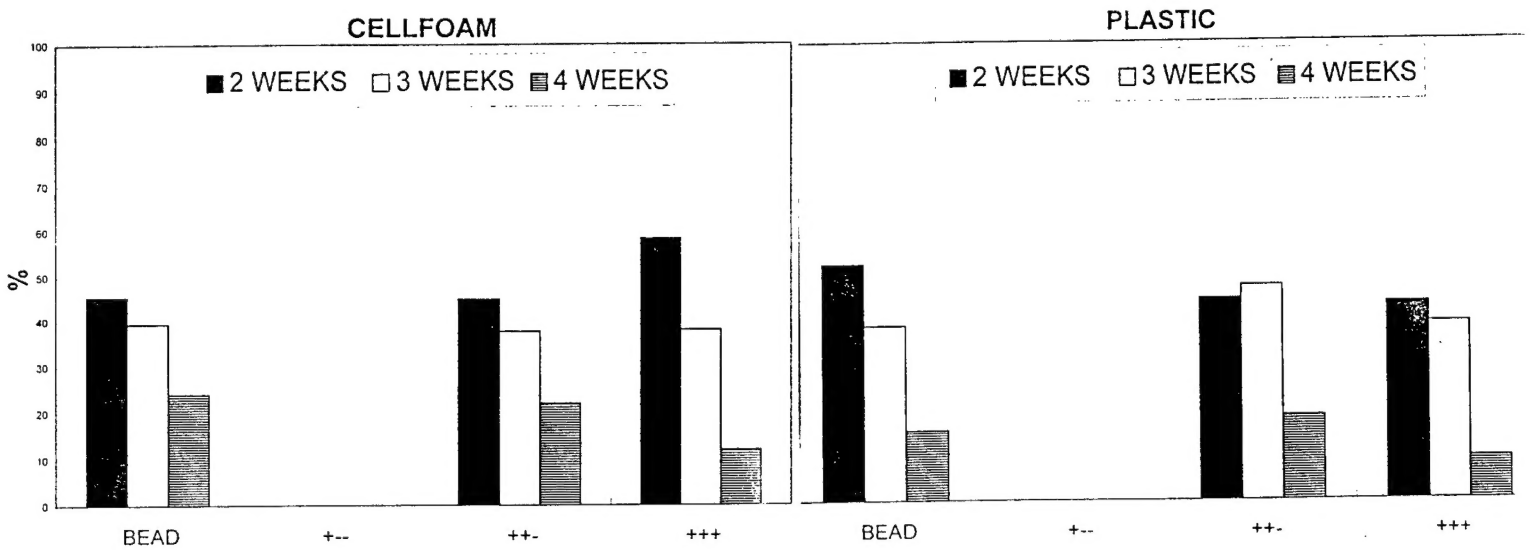
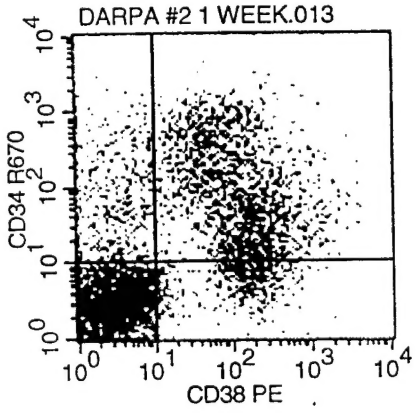


Figure 5. Primary flow cytometry data illustrating the presence of $CD34^+38^-$ cells in erythropoietin-supplemented Cellfoam cultures.



Patient ID:
Gated Events: 6231
X Parameter: FL2-H CD38 PE (Log)
Quad Location: 10, 10

Gate: G1
Total Events: 10000
Y Parameter: FL3-H CD34 R670 (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	517	8.30	5.17	3.91	3.09	212.83	73.01
UR	2004	32.16	20.04	223.94	117.63	252.95	93.52
LL	3086	49.53	30.86	3.36	2.78	3.41	2.92
LR	624	10.01	6.24	182.96	105.96	6.05	5.44

Thrombopoietin cultures: The trend of decreasing $CD34^+$ content detected in the erythropoietin cultures also emerged in the thrombopoietin cultures over the 4 week evaluation period (Figure 6). The percent $CD34^+$ again was similar between Cellfoam and plastic at 2 and 3 weeks. Further, similar to the observations of erythropoietin cultures, at 4 weeks the $CD34^+$ content was markedly enriched in Cellfoam cultures of bead purified cells and $CD34^+CD38^+61^-$ and $CD34^+CD38^+61^+$ sorted cells, relative to plastic (Table IV). Overall, Cellfoam retained 30-263% more $CD34^+$ cells than plastic at 4 weeks. Strikingly, this preservation occurs in the presence of high levels of cytokines that typically mediate stem cell differentiation. This again indicates that the immature CD34 compartment is being retained to a greater degree in Cellfoam than in plastic, an important attribute with regard to generating sustained differentiation systems.

Figure 6. Percent $CD34^+$ cells in thrombopoietin-supplemented Cellfoam and plastic cultures. +-- = $CD34^+38^-61^-$; ++- = $CD34^+38^+61^-$; +++ = $CD34^+38^+61^+$

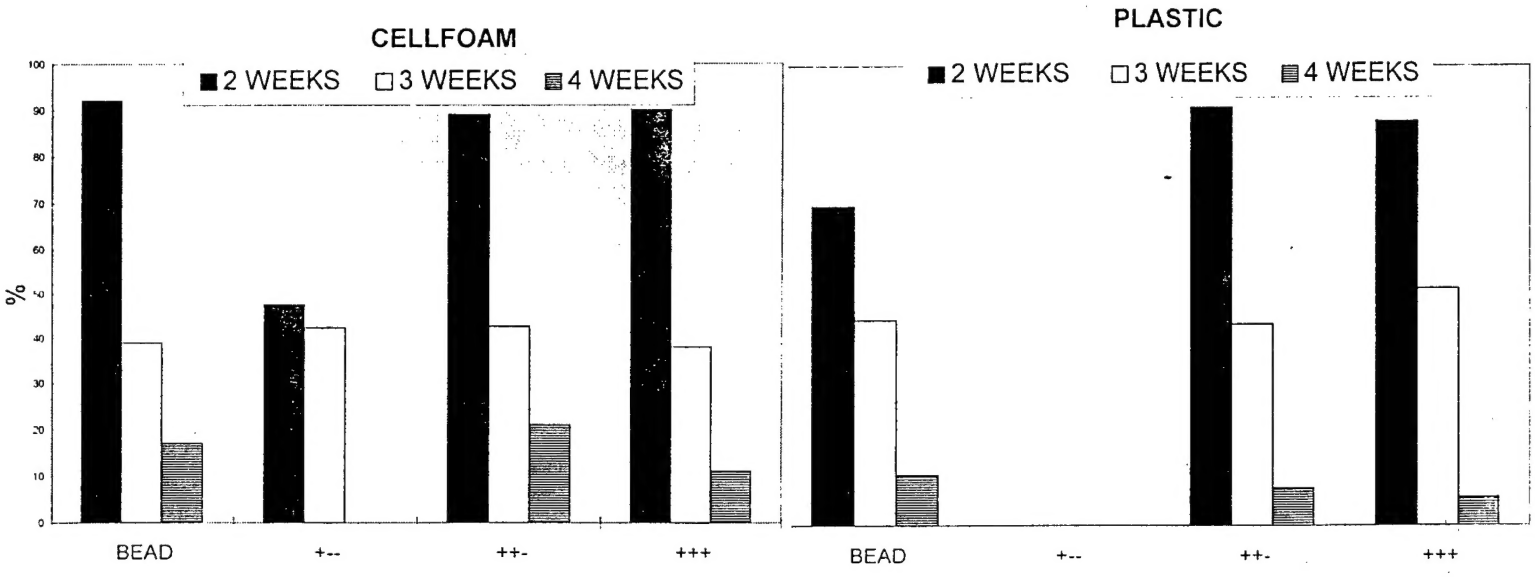
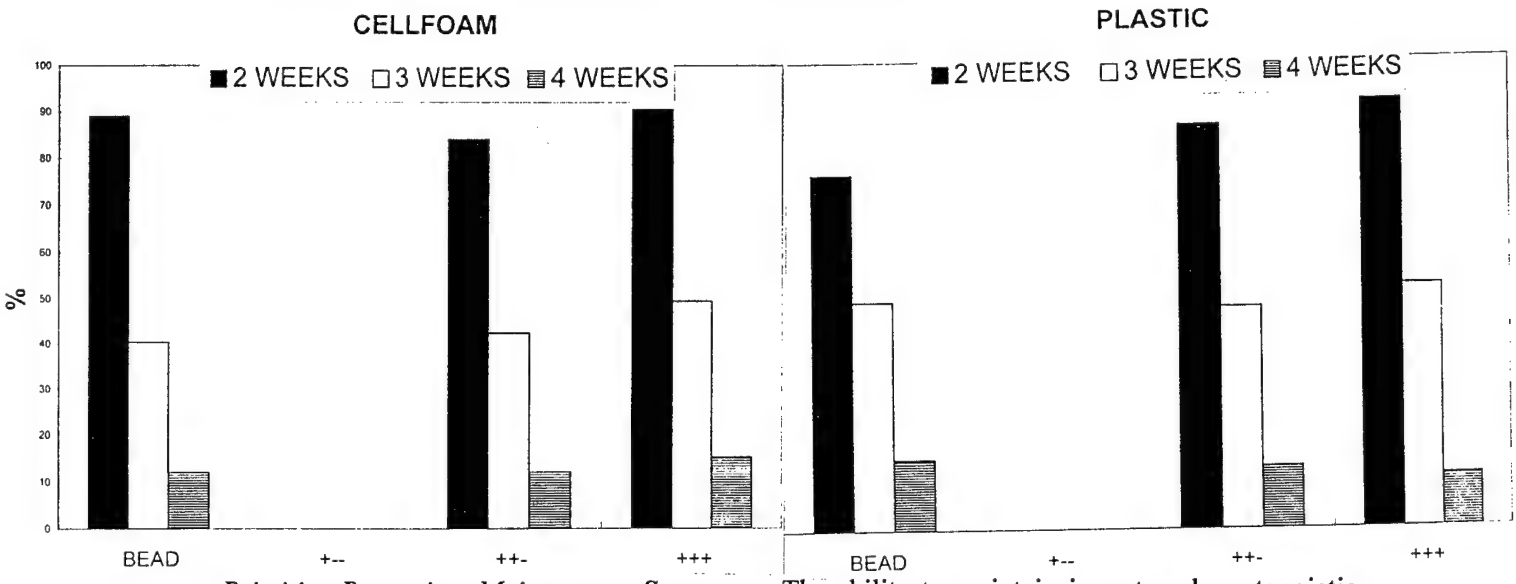


Table IV. Percent of cells expressing the CD34 antigen in 4 week thrombopoietin-supplemented Cellfoam and plastic cultures.

	Cellfoam	Plastic
bead purified	17	11
$CD34^+CD38^+61^-$	21	8
$CD34^+CD38^+61^+$	11	8

GMCSF cultures: HPCs cultured in the presence of IL-3, SCF and GMCSF exhibited a pattern of $CD34^+$ depletion over time that was very similar to the patterns observed with erythropoietin and thrombopoietin (Figure 7). Percentages of $CD34^+$ cells again were very similar between Cellfoam and plastic cultures at 2 and 3 weeks. However, contrary to Epo and Tpo cultures, Cellfoam cultures supplemented with GMCSF did not exhibit any appreciable increases in CD34 content relative to plastic cultures at 4 weeks. The basis for the differential response of HPCs to the different cytokines is unclear.

Figure 7. Percent $CD34^+$ cells in GMCSF-supplemented Cellfoam and plastic cultures.
+-- = $CD34^+38^-33^-$; ++- = $CD34^+38^+33^-$; +++ = $CD34^+38^+33^+$



Primitive Progenitor Maintenance Summary: The ability to maintain immature hematopoietic progenitors in the presence of differentiation cytokines is of considerable importance in the generation of a long-lived blood cell production system. Collectively, the work described above demonstrates that even in the presence of high levels of such cytokines, a proportion of cells detected within the culture systems maintains at least some immature phenotypic characteristics. In particular, cells expressing the CD34 antigen could be detected at time-points as long a 4 weeks in the presence of differentiation cytokines. Interestingly, the percentage of $CD34^+$ cells, and in some cases $CD34^+38^-$ cells, was consistently greater in Cellfoam than in plastic at the latest time-point examined (4 weeks). This overall observation is consistent with previous data demonstrating that Cellfoam preferentially maintains and expands immature hematopoietic cells in the absence of supplemented cytokines (see Phase I proposal and below, Additional Work). This encoring property appears to extend to conditions in which certain cytokines have been added exogenously. This potential is important because it suggests that not only may primitive multipotent progenitors be preserved and/or expanded in a cytokine-free Cellfoam compartment.

Operating from the principle that both stem cell maintenance and differentiation are desirable in a blood cell production system, a multi-chamber Cellfoam system can be envisioned in which stem cells are maintained in an initial cytokine-free chamber from which slightly more mature cells, perhaps with long-lived traits, are removed and transferred to and preserved in an intermediate Cellfoam chamber in which particular cytokines have been added to begin the induction of lineage targeted differentiation. Subsequently, differentiating progeny could be removed to a third compartment wherein they would be subjected to terminal differentiation, perhaps accomplished without Cellfoam as there is not as yet a compelling reason to use the device in terminal differentiation steps. Thus, Cellfoam offers an innovation in achieving the entire gamut of long-term stem cell survival, maintenance of multipotency and differentiation. Because Cellfoam offers the benefit of maintaining long-lived immature progenitors, it will be of obvious benefit in supporting a high-throughput system for scale-up, particularly when augmented with accessories such as a perfusion system (described later).

Lineage Positivity:

In general, a substantial body of evidence has been developed which supports the view that lineage progression of HPCs can be broadly appreciated under the following paradigm:

$$CD34^+38^-lin^- \text{---> } CD34^+38^+lin^- \text{---> } CD34^+38^+lin^+ \text{---> } CD34^-38^-lin^-$$

Operating from this model, each task evaluated the acquisition of phenotypic markers indicative of progressive differentiation/maturation in the presence of the respective cytokine cocktails. We have already shown that 1) Cellfoam yields similar cell numbers (i.e. expansion) as plastic at many time-points and 2) $CD34^+$ content is maintained to a greater extent in Cellfoam than in plastic in the presence (and absence) of cytokines. To compare the extent to which lineage progression occurs in Cellfoam versus plastic, the presence of $CD34^+CD38^+lin^+$ and $CD34^-CD38^-lin^-$ cells was determined in the two systems.

Erythropoietin cultures: As shown in Figure 8, while $CD34^+CD38^+71^+$ numbers were approximately equal in erythropoietin-supplemented Cellfoam and plastic cultures at each time-point (with the exception of the 4 week bead time-point), Cellfoam universally yielded higher numbers of $CD34^-CD38^-71^-$ cells in the bead separated, $CD34^+CD38^+71^-$ and $CD34^+CD38^+71^+$ cultures at the 3 and 4 week time-points. This indicates that a larger percentage of cells reached later stages of maturation in Cellfoam than in plastic. Together, the drop in the $CD34^+CD38^+71^+$ population from 2 to 4 weeks, coupled with the increase in $CD34^-CD38^-71^-$ content is indicative of the progressive maturation of cells along the erythroid lineage.

Thrombopoietin cultures: In thrombopoietin cultures, the percentage of $CD34^+CD38^+61^+$ cells at 2 weeks was greater in Cellfoam than in plastic, and while it declined in both systems at 3 and 4 weeks, the total percentage was similar between Cellfoam and plastic at 3 and 4 weeks (Figure 9). The frequency of $CD34^-CD38^-61^-$ cells, however, was greater at 3 and 4 weeks in Cellfoam than in plastic and gradually increased with time. As in the Epo supplemented cultures, this finding points to the superior progressive differentiation of cells along the thrombocyte lineage in Cellfoam cultures. Compared with the erythropoietin cultures, the drop-off in the $CD34^+38^+lin^+$ content and the accumulation of the $CD34^-38^-lin^-$ cells in the Tpo cultures was much more rapid, suggesting that this cytokine may act more quickly than Epo on later stage progenitors.

GMCSF cultures: The trend observed in the Epo and Tpo cultures again was detected in the GMCSF cultures. In both Cellfoam and plastic, the relative percent of $CD34^+CD38^+33^+$ cells declined over 4 weeks, with a large drop occurring between 2 and 3 weeks. No clear benefit of Cellfoam versus plastic was found in the maintenance of the $CD34^+CD38^+33^+$ population. Conversely, Cellfoam was demonstrated to have substantially greater ability to promote the development of $CD34^-CD38^-33^-$ cells over 2 to 4 weeks in the presence of GMCSF, again suggestive of progressive differentiation. Further, at 2 and 3 weeks, high percentages of cells expressed either CD14 and CD 66, markers which are representative of mature monocyte and granulocyte differentiation, respectively.

Lineage Generation Summary: While they enact specific and separable differentiation pathways, the cytokines analyzed above demonstrated several similarities in their effects on hematopoietic progenitors. First, over time the percentage of $CD34^+CD38^+lin^+$ cells decreases while the percentage of $CD34^-CD38^-lin^-$ cells increases. This suggests that in each case progressive maturation of hematopoietic cells was achieved (as supported by the increase in $CD34^-38^-lin^-$ cells), a conclusion that is supported by the ongoing phenotypic development detected by microscopy (see morphologic evaluation below). Interestingly, in Cellfoam at least, differentiation is supported while some $CD34^+$ (including $CD34^+38^-$) content is preserved. This indicates that maturation and maintenance can occur in the same device in the presence of specific cytokines, a finding that can be used integrally in the final development of the Cellfoam blood cell differentiation system.

Figure 8. Percent $CD34^+CD38^+71^+$ and $CD34^-CD38^-71^-$ produced in erythropoietin-supplemented Cellfoam and plastic cultures over 2-4 weeks. The starting population (i.e. bead purified or sorted subset) is listed on the X-axis. CF = Cellfoam; PL = Plastic.

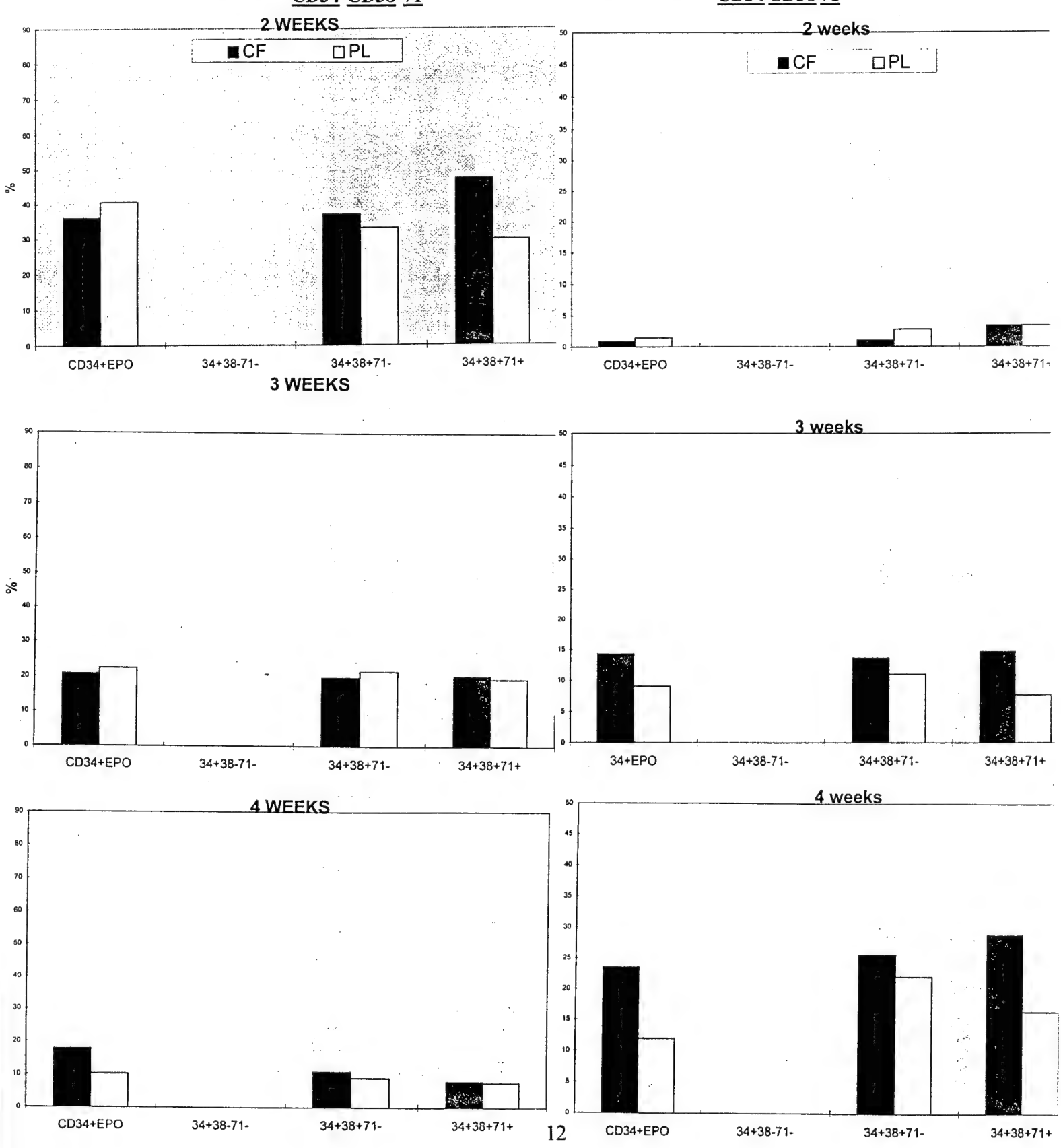


Figure 9. Percent $CD34^+CD38^+61^+$ and $CD34^-CD38^-61^-$ produced in thrombopoietin-supplemented Cellfoam and plastic cultures over 2-4 weeks. The starting population (i.e. bead purified or sorted subset) is listed on the X-axis. CF = Cellfoam; PL = Plastic.

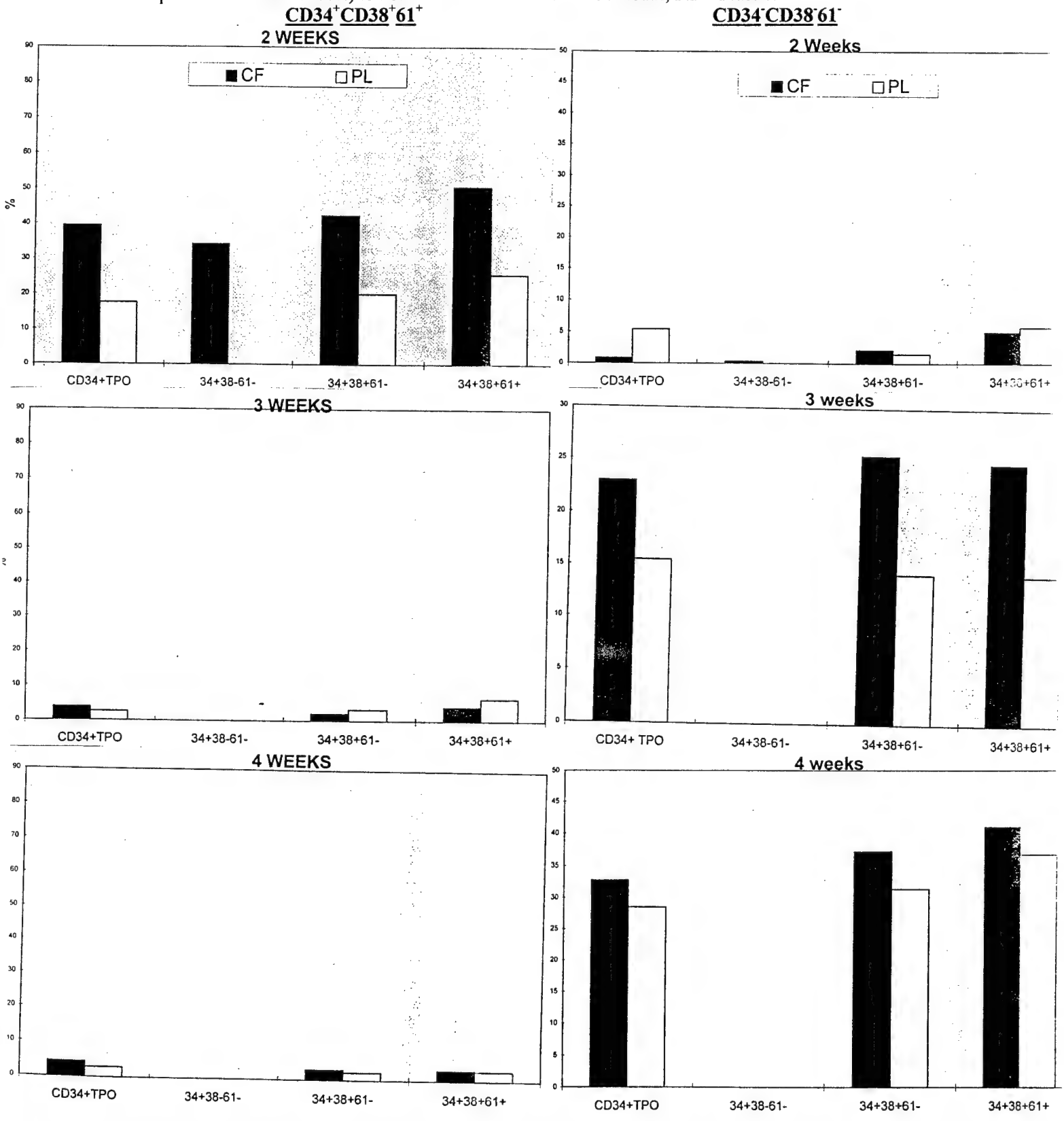
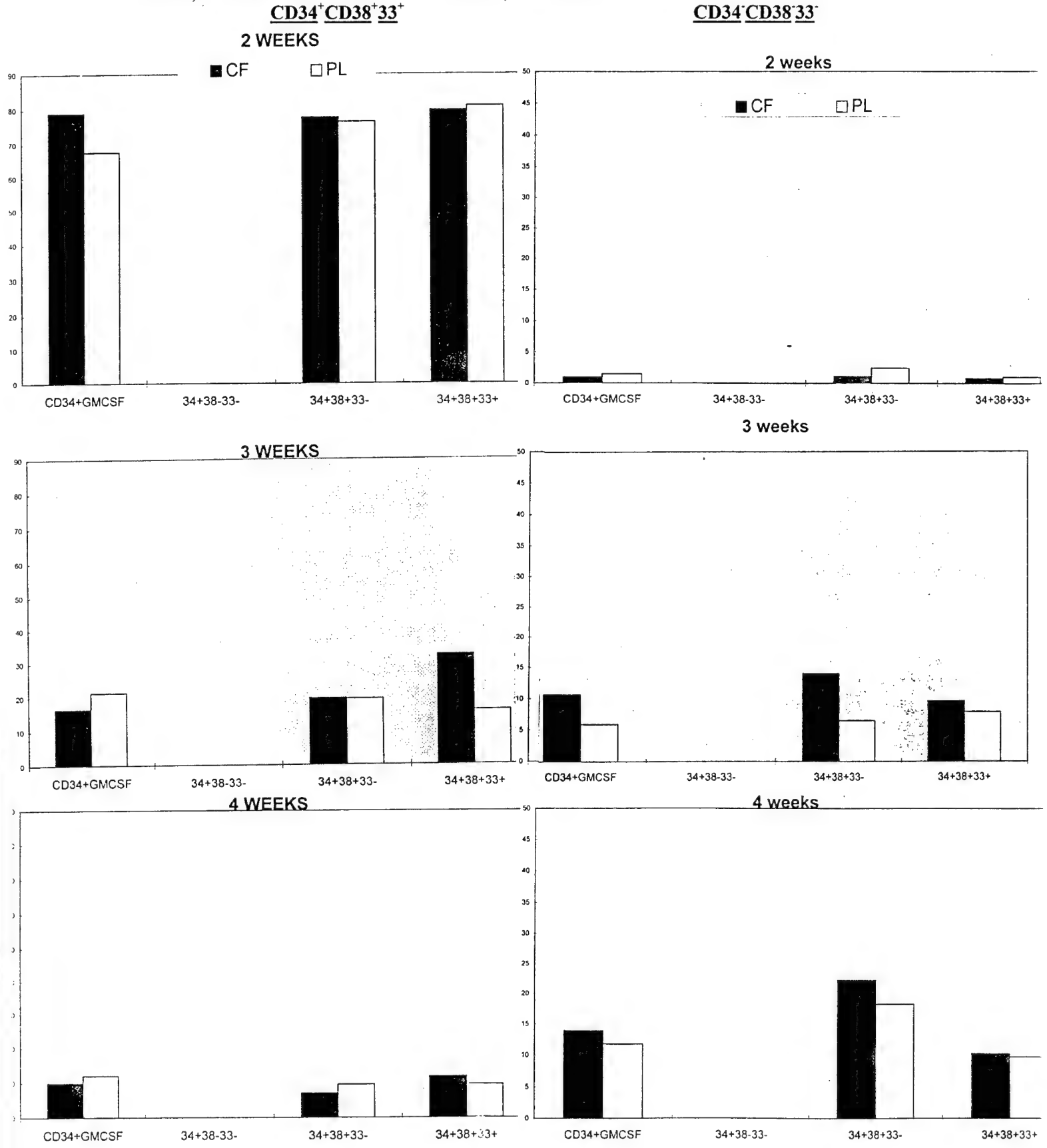


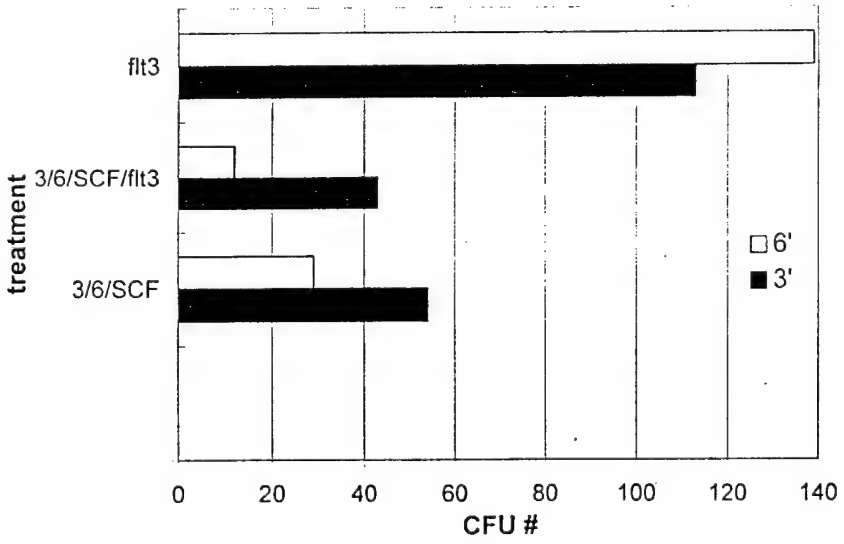
Figure 10. Percent $CD34^+CD38^+33^+$ and $CD34^-CD38^-33^-$ produced in GMCSF-supplemented Cellfoam and plastic cultures over 2-4 weeks. The starting population (i.e. bead purified or sorted subset) is listed on the X-axis. CF = Cellfoam; PL = Plastic.



CD34 Content in Cytokine-Free v. Cytokine-Augmented Cultures:

Because the studies described above pointed to the possibility that $CD34^+$ cells could be maintained in the presence of differentiation cytokines, a study was performed to directly compare the CD34 content in cytokine-free and cytokine-supplemented cultures in Cellfoam and plastic. The aim of this experiment was two-fold: 1) to determine the effect of cytokine addition on CD34 content and 2) to begin to combine the sequential integration of cytokine-free culture with cytokine-supplemented culture. In these studies, hematopoietic progenitors from human bone marrow were separated by anti-CD34 immunomagnetic beads and cultured in Cellfoam or plastic for three weeks in the absence of supplemented cytokines. After this three week culture period, cytokines were added as described above for an additional two weeks. Specifically, the cytokines added were IL3 + SCF, or IL3 + SCF + either erythropoietin, thrombopoietin or GMCSF. The concentrations of Epo, GMCSF and Tpo were the same as used in the differentiation cultures described above. However, the concentrations of IL3 and SCF were lowered to 5 ng/ml based on the premise that high levels of cytokines may rapidly exhaust the stem cell population. One culture was kept as a control, in duplicate, and received no cytokines at any time. Following the two week culture period in the presence of cytokines (except for the control), adherent and non-adherent cells were isolated, pooled and evaluated for CD34 content. The results of a representative trial are shown in Figure 11. The results affirm several conclusions predicted by the collective experiments described above. First, in all cases, Cellfoam led to greater preservation of $CD34^+$ cells than plastic. The improvement in $CD34^+$ maintenance ranged from 1.3 to 2 fold, and was detected in the cytokine-free control and all cytokine-supplemented cultures. Second, while $CD34^+$ cell were detected upon the addition of cytokines (with relatively low levels), the frequency dropped by about two fold compared to unsupplemented cultures. Interestingly, the frequency of $CD34^+$ cells was similar in IL3/SCF, Epo, Tpo and GMCSF cultures, suggesting that both the absolute attrition and the rate of decline in CD34 content is a general phenomenon of cytokine supplementation. This observation reiterates the effect of cytokine supplementation on hematopoietic stem cell cultures noted by others in the field specifically showing that cytokines negatively effect the survival of long-lived multipotent progenitors. Thus, one conclusion from this work and the work described above is that when cytokines are added to stem cell cultures some degree of CD34 content can be preserved, but the frequency of these immature cells is diminished relative to unsupplemented cultures. This again highlights the attractiveness of a multi-chamber Cellfoam system in which different chambers are designed to achieve distinct effects on the stem cells (e.g. maintenance v. differentiation) which would make expedient use of Cellfoam's unique capability to support long-term survival of multipotent progenitors in the absence of cytokines.

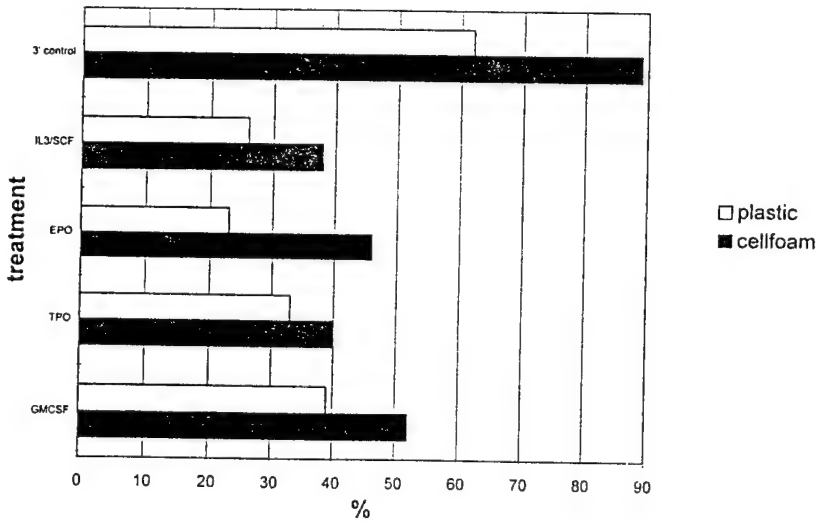
Figure 11. CFU activity of cells in cytokine-supplemented v. cytokine-free Cellfoam and plastic cultures.



Effects of Cytokines on Stem Cell Multipotency:

The experiments described above which comprised the focus of the DARPA Phase I project illuminated numerous salient features of hematopoietic stem cell culture in Cellfoam and of the effects of cytokine supplementation on stem cell survival and immaturity. However, one critical feature of stem cell biology, the ability to generate colonies in standard assays (a measure of multipotency and function), was not specifically addressed in these studies. While $CD34^+$ cell content provides clues about stem cell maturity/immaturity and may be used to infer the degree of multipotency, it is not a direct measure of colony-forming activity. To fully evaluate the effects of cytokine supplementation on stem cells, an experiment was performed in which hematopoietic progenitors were cultured in the presence of low levels of cytokines for three and six weeks in Cellfoam and subsequently isolated from the cultures and placed in methylcellulose colony formation assays. Because it was anticipated that the use of high concentration cytokines would rapidly deplete colony-forming ability over six weeks, the specific levels of cytokines used were approximately a log-fold lower than those used in the differentiation experiments described above (IL3 and IL6 @ 5 ng/ml, SCF @ 10 ng/ml, Flt-3 @ 100 ng/ml). Figure 12 shows results from a representative experiment. The figure illustrates the predominant effect of cytokines on stem cells: the depletion of colony forming activity over time. In particular, the combinations of IL3/IL6/SCF/flt-3-L and IL3/IL6/SCF led to a progressive reduction in colony forming activity, evident in the histogram as a decrease in total colony content from 3 to 6 weeks. However, an interesting pattern was detected with flt-3 ligand alone. With this cytokine, the colony forming activity remained substantially higher than with other cytokines; in fact colony activity increased between 3 and 6 weeks. The increase in colony activity raises the possibility the certain cytokines, such as flt-3 ligand, may promote colony forming activity. The impact of such cytokines on the long-term survival and multipotency of immature progenitors is not known and will be addressed in subsequent experiments.

Figure 12. Colony forming activity in cytokine-supplemented Cellfoam cultures at 3 weeks.



Histologic Morphology:

To evaluate the morphology of the cells produced in the cytokine-supplemented cultures, histologic analyses were performed in which cells isolated from the Cellfoam and plastic culture systems were cytospun onto glass slides and stained with Diff-Quik. The slides were then evaluated by light microscopy by two blinded investigators who evaluated relative cell frequencies and ratios (e.g. erythroid, myeloid and platelet lineages), overall cell abundance, the degree of 'normal' morphology and the rate of dead cells.

Erythropoietin cultures: Erythropoietin augmented cultures were evaluated by at 2, 3 and 4 weeks for the presence of differentiating erythrocyte progeny. In all cultures, cells with phenotypic similarity to red cell precursors could be detected. At 2 weeks, cells harvested from Epo supplemented cultures of $CD34^+$ bead purified cells or of $CD34^+38^+71^+$ cells in Cellfoam contained a high percentage erythrocyte precursors characterized as large cells with prominent nuclei and basophilic cytoplasm (28% and 26%, respectively). Plastic cultures of these cell subsets exhibited a lower percentage of such cells (11% and 14%). The percentage of cells resembling immature erythrocyte progenitors from prorubricytes to metarubricytes cells increased over 2 to 3 weeks. In the bead purified population, this percentage increased to 32% in Cellfoam and 19% in plastic. In the $CD34^+38^+71^+$ subset, the percentage of meta- and pro-rubricytes in Cellfoam and plastic was equal at 25%. By 3 weeks, many cells had become smaller and had acquired a brown pigment indicative of hemoglobin content in immature rubriblasts (see Figure 13a for Cellfoam). A greater number of brown-pigmented cells was detected in Cellfoam than in plastic. This trend continued through four weeks and is suggestive of progressive differentiation of red cell precursors in Epo-supplemented Cellfoam cultures and to a lesser extent in plastic. Importantly, at four weeks in Cellfoam, apparently mature red cells could be detected in the bead purified cultures. This encouraging finding was not recapitulated in any plastic cultures. Interestingly, a proportion of the cells exhibited traits of polymorphonuclear cells, which may reflect the promiscuity of the cytokines used and which suggests that simultaneous differentiation of red cells and white cells may be possible *in vitro*.

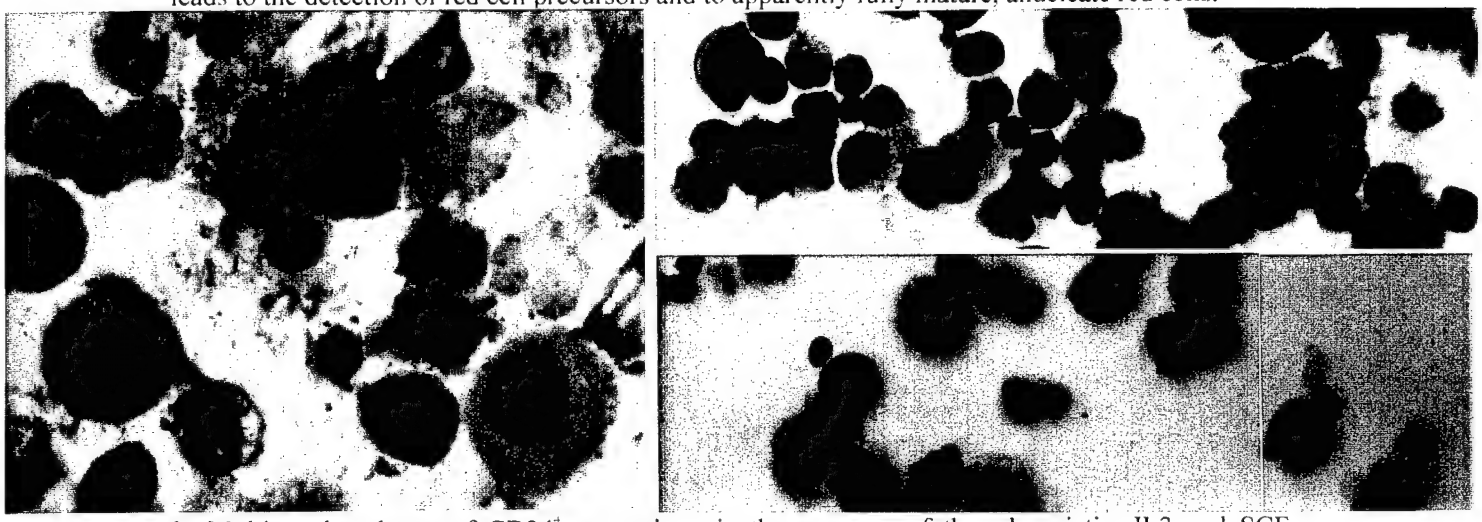
Thrombopoietin cultures: Similar histologic analyses of cells from thrombopoietin supplemented cultures revealed progressive cell differentiation as well. The percent of cells exhibiting qualities of maturing megakaryocytes (defined as large, non polymorphonuclear cells, with vacuoles, feathered-edge cytoplasm, and/or multiple nuclei) was again consistently greater in Cellfoam at 2 and 3 weeks (Figure 13b). In both the Cellfoam and plastic culture systems, multinucleate cells could be detected by 3 weeks in the $CD34^+38^+61^+$ subset. Fewer of these cells were observed in the bead purified populations. As a defining feature of megakaryocyte ontogeny is the development of cells with 4n through 32n or 64n DNA content, or polyploidism, the detection of multinucleate cells is consistent with the ongoing generation of platelet precursors. These cultures also showed the presence of red cell progenitors, reaffirming the promiscuous role of Tpo in RBC and platelet differentiation previously been shown by others. Thus, the inclusion of the thrombopoietin cocktail appears to lead to the acquisition of phenotypic and molecular markers of platelet development as *in vivo*, and may have relevance for RBC production as well.

GMCSF cultures: Morphologically, by 2 weeks cells harvested from GMSCF/cytokine supplemented cultures in Cellfoam were found to contain large cells with polymorphic nuclei and vacuolated cytoplasm containing basophilic and acidophilic granules. The range of cells sizes was diverse, as was their granularity (Figure 13c). Granularity included moderate and strong eosinophilic and deep basophilic appearances which suggests the identification of neutrophilic, eosinophilic and basophilic granulocytes. Direct evidence for the presence of monocytes was inferred by the presence of cells with small, uniform spherical nuclei with abundant, light eosinophilic cytoplasm containing no granules. These presence of these cells is in agreement with the CD14 and CD66 antigen expression patterns observed by flow cytometry. In both Cellfoam and plastic, the percentage of cells with these characteristics increased from 2 to 3 weeks and then leveled off in week 4. Thus, differentiation of white cell precursors in

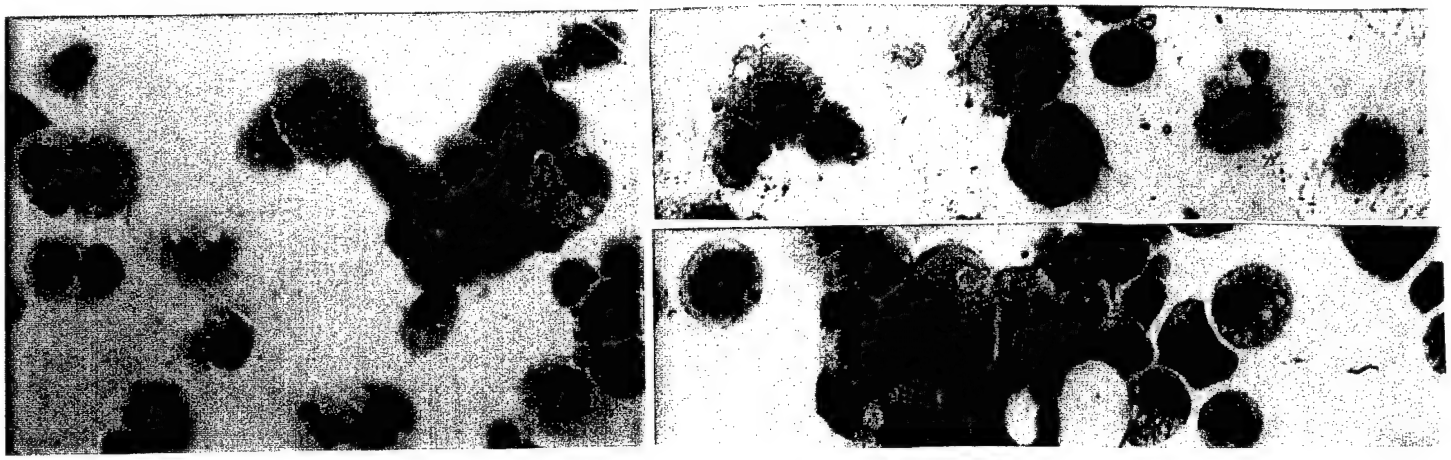
GMCSF-supplemented Cellfoam cultures is supported by both phenotypic and molecular marker data.

Figure 13. Histologic morphology of $CD34^+$ HPCs cultured in Cellfoam.

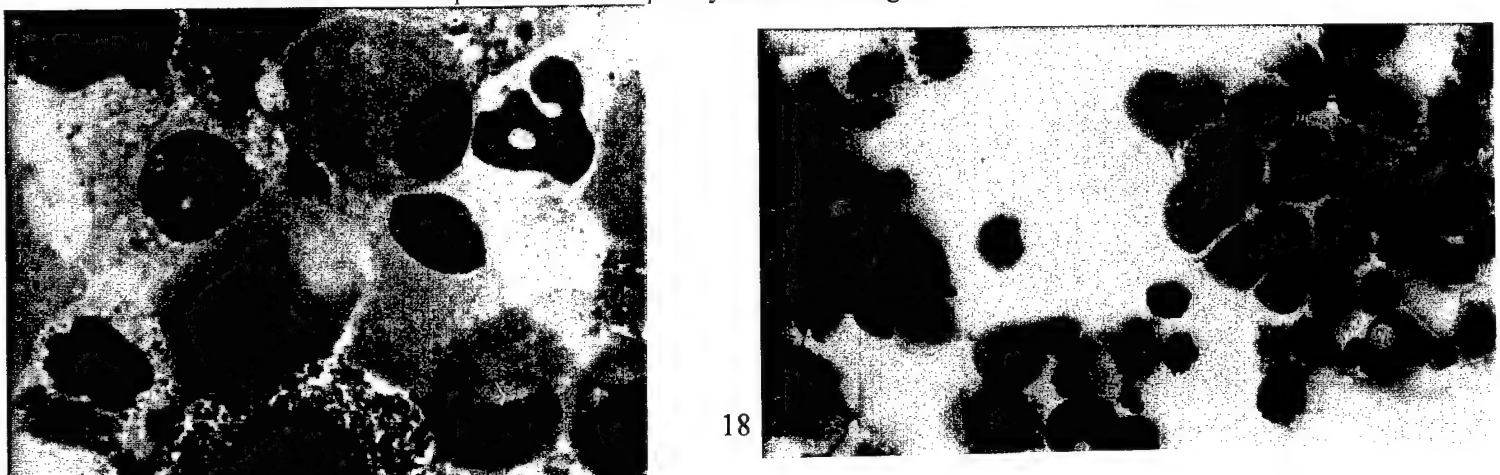
a. Culture of bead separated HPCs over four weeks in the presence of erythropoietin, IL3 and SCF leads to the detection of red cell precursors and to apparently fully mature, anucleate red cells.



b. Multi-week cultures of $CD34^+$ progenitors in the presence of thrombopoietin, IL3 and SCF contain primitive cells with features of the megakaryocyte lineage, including large size, some cytoplasmic granularity with eosinophilic affinity, and feathered cytoplasm.



c. The addition of GMCSF (with IL3 and SCF) to HPC cultures yields myeloid cells with divergent traits consistent with the presence of multiple myeloid sub-lineages.

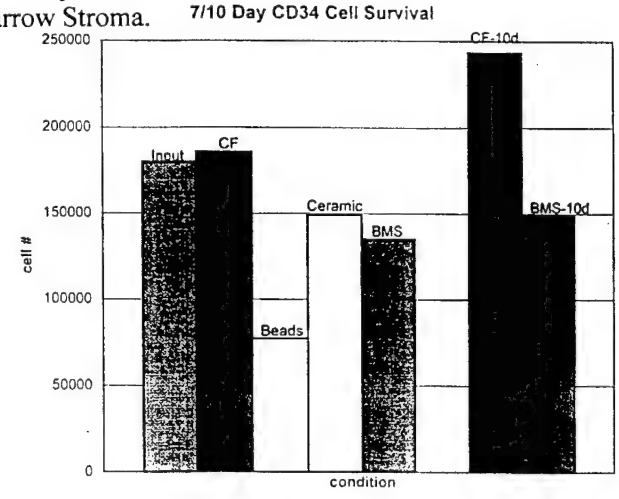


Additional work:

Cellfoam Enables HPC Survival Over Extended Periods and Expands HPC Content: While the emphasis of the Phase I DARPA project focused on cytokine-mediated differentiation of targeted cell types, continued progress was made on the extended preservation of HPCs in cytokine-free Cellfoam cultures. These studies extended preliminary short-term (~ 1-2 weeks) work and demonstrated that $CD34^+$ HPCs cultured in fibronectin-coated Cellfoam *without cytokine supplementation* exhibit enhanced survival and marked enrichment compared to parallel control cultures using bone marrow stroma, fibronectin coated plastic dishes, or other 3-D substrates, including a hydroxyapatite ceramic and dextran microbeads. Additional three dimensional devices were examined to gain insight into the basis of the effects of materials and geometry on HPC survival *ex vivo*. Additional culture time-points of 1, 1.5, 3, and 6 weeks were also analyzed to obtain a representative profile of the $CD34^+$ cell counts over time.

In the control systems, including other 3-D materials, without cytokines an input population of 180,000 $CD34^+$ cells declines in number over time. This loss of HPCs in control cultures supports general observations documenting an inability of conventional systems to sustain HPCs in the absence of cytokines. Conversely, the input HPC population not only *survives* in Cellfoam but is *expanded.* At 1 and 1.5 weeks, $CD34^+$ cells in control systems had already begun to decline, ranging from 82% of input cell number (ceramic) to less than 40% of input numbers (dextran beads). Representative results are shown in Figure 14a. Plastic cultures performed similarly to the bone marrow stroma system (not shown). Conversely, by one week $CD34^+$ cell counts in Cellfoam were 2.5-3 fold higher than other systems analyzed and had increased by about 5-6% over input numbers. By 10 days, $CD34^+$ expansion in Cellfoam had further increased to 30-35% above input numbers. This increase in cell number was reproducible and occurred in *the absence of supplemented cytokines*. Further, a population of cells that retained the immature $CD34^+CD38^-$ phenotype and which was enriched in Cellfoam compared to control bone marrow stroma cultures could be detected at 1, 1.5 and 3 weeks (see Fig. 14b). The increase in $CD34^+$ content in Cellfoam was especially pronounced at three weeks: whereas $CD34^+$ numbers continued to decline in control cultures, $CD34^+$ cells continued to expand in Cellfoam to over 380,000 (and up to 500,000), an increase from input numbers of 200-300% and a 3-5 fold enrichment over $CD34^+$ numbers in control systems (Fig. 15). At 6 weeks, $CD34^+$ numbers remained 200-250% greater than input numbers and 12 to 16 fold higher than controls. The findings at 6 weeks illustrate the continued relative enhancement of HPC survival in Cellfoam versus conventional systems. Importantly, HPCs expanded in Cellfoam retained both $CD34^+38^-$ fractions (Figure 14b) and multipotency as a bulk population, as described below.

Figure 14. Survival of HPCs in Cellfoam v. control systems.
a) Representative 7 day $CD34^+$ cell survival; 10 day results are also presented for Cellfoam and Bone Marrow Stroma.



b) Flow cytometry at 3 and 6 weeks in Cellfoam and Bone Marrow Stroma

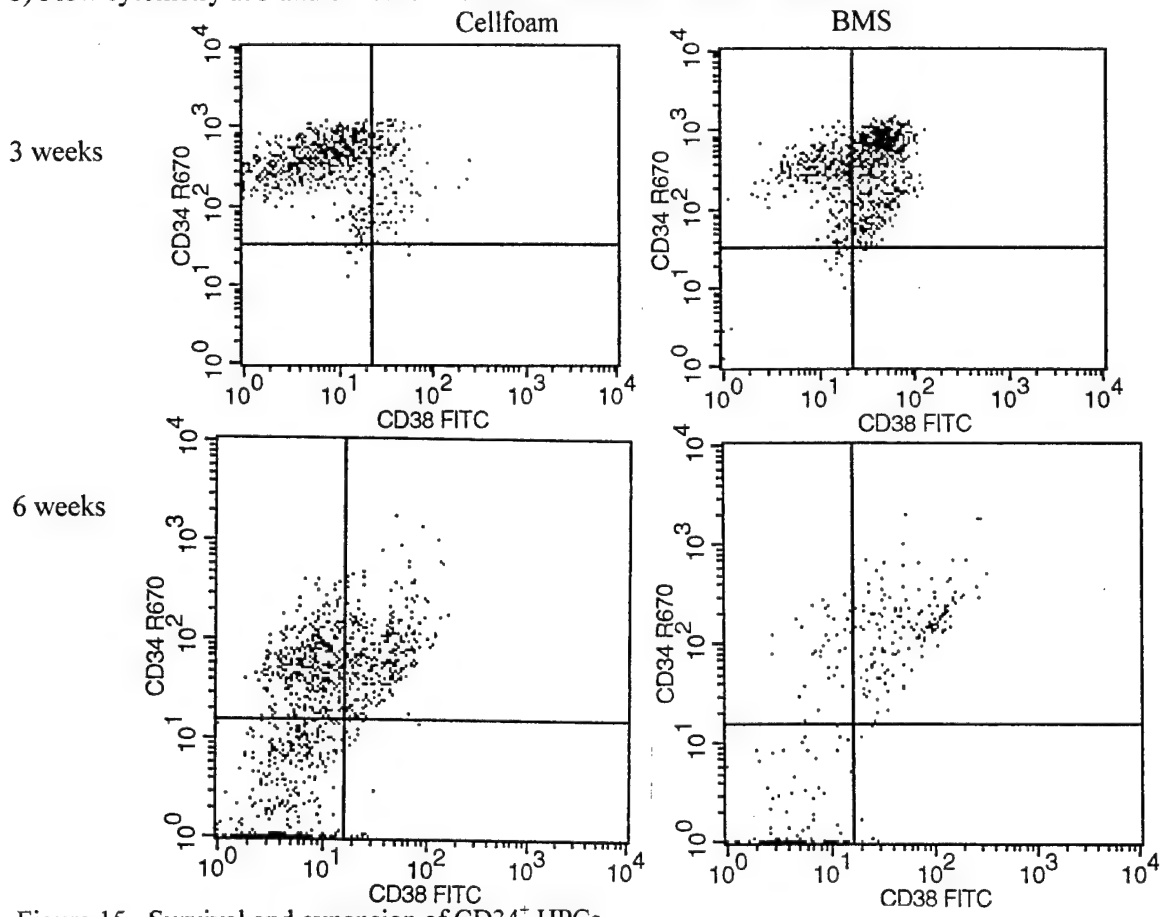
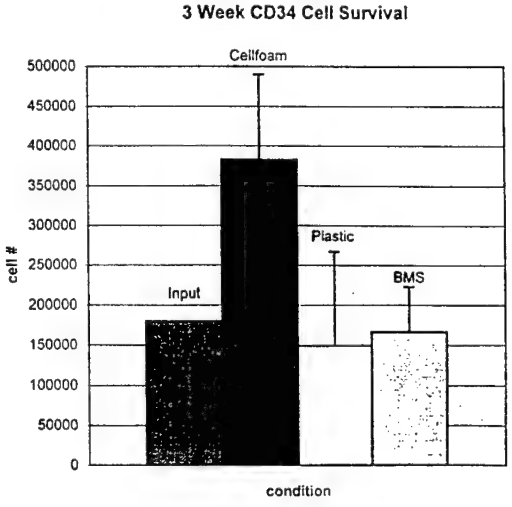
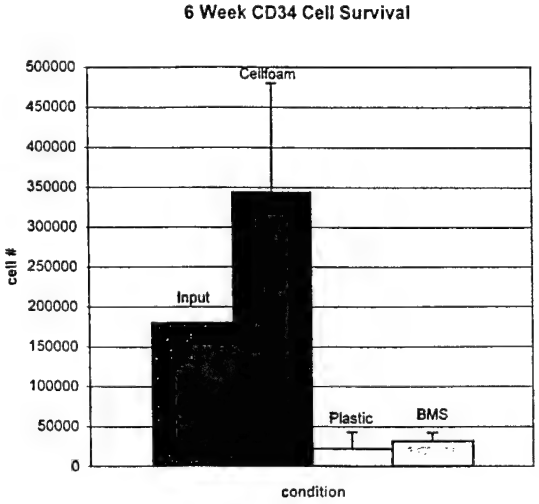


Figure 15. Survival and expansion of $CD34^+$ HPCs in Cellfoam v. controls after 3 and 6 weeks.

a) 3 weeks



b) 6 weeks

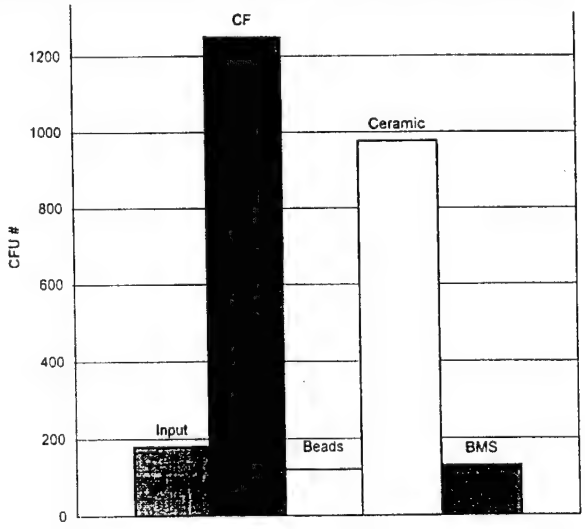


Long-term Culture Conclusion: Culture of $CD34^+$ hematopoietic progenitors in Cellfoam in the absence of cytokines not only enables long-term survival of these cells but also fosters *expansion* of up to 300% over initial input population *in absence of cytokines* in less than 3 weeks. CD34 populations greater than input numbers are sustained through 6 weeks. The cytokine-free expansion of HPCs under static conditions has not been reported for other systems. Culture in Cellfoam also yielded up to 16 times more $CD34^+$ cells than control cultures. The relative abilities of the different systems to support HPC survival provide insight into properties of the devices that affect HPC survival. The findings suggest that several aspects of the culture environment may affect HPC culture, including three dimensionality, material composition, porosity and the presence of supporting stromal cells. The improvements over monolayer plastic observed in bone marrow stroma, ceramics and beads suggests that stromal cells, porous three dimensionality and inert, biocompatible three dimensionality may improve HPC survival *ex vivo*. The presence of all of the properties in Cellfoam may contribute to its overall improved performance in HPC culture.

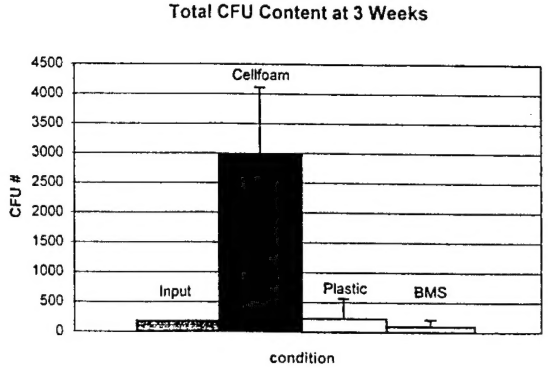
Improved Maintenance of $CD34^+$ HPC Multipotency in Cellfoam: To extend the numerical cell data in cytokine-free cultures described above, cells isolated from cytokine-free culture devices at various time-points were placed into methylcellulose and T-cell assays as a measure of function. HPCs from Cellfoam cultures retain RBC and WBC colony forming ability to a greater extent than cells from parallel control cultures, including other three dimensional substrates. The enhanced colony-forming ability of Cellfoam-isolated cells relative to cells from other systems is evident by 1 week. In BMS (Figure 16a) and plastic controls (not shown), CFU content decreased from input capabilities. This reduction was similar to the decline in overall $CD34^+$ cell counts in these systems by one week. Interestingly, at 1 week of culture both Cellfoam and the ceramic showed expanded total CFU content relative to input capabilities. In particular, CFU content in Cellfoam and ceramic devices were enriched approximately 7 fold and 5.5 fold, respectively. The ceramic had a colony-per-isolated cell frequency similar to Cellfoam. However, because more cells are obtained in Cellfoam, the absolute number of colonies in Cellfoam was greater than in the ceramic. In all cultures, both CFU-GM and BFU-E were evaluated; the myeloid:erythroid ratio in all conditions was approximately 2:1. At subsequent weeks, the CFU content in Cellfoam continued to increase and was markedly enhanced relative to control systems. At 3 weeks, Cellfoam cultures yielded up to 31 times as many colonies as control BMS and plastic cultures, representing an increase of 16 fold over input capabilities. This enrichment over input cells was sustained through 6 weeks. Further, by six weeks in conventional BMS or plastic cultures, HPCs had lost essentially all of their colony-forming ability; at this time, HPCs from Cellfoam cultures had over a 1000 fold greater capacity to produce colonies over cell isolated from BMS and plastic-fibronectin cultures (Fig. 16c). These cells also retained bulk capabilities of differentiating along all lineages. This sustained increase in colony-forming ability parallels the expansion of HPCs in Cellfoam.

Figure 16. CFU ability of HPCs isolated from Cellfoam and control cultures

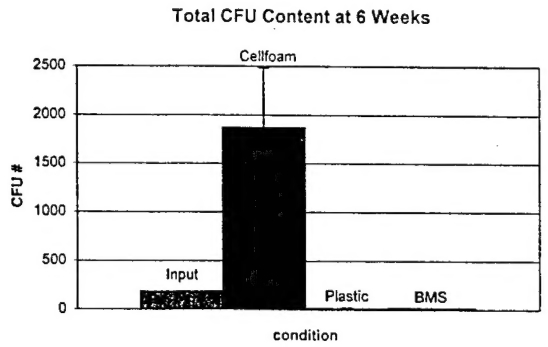
a) 1 week



b) 3 weeks



c) 6 weeks



Enhanced T cell lymphopoiesis in Cellfoam: To evaluate the T cell forming ability of cells isolated from long-term cytokine-free cultures, we performed an *in vitro* T-lymphopoiesis assay, prepared as described above. After termination of cultures at 3 and 6 weeks, an aliquot of the combined adherent-non-adherent fractions were co-cultured with primary fetal thymic stroma. This culture system facilitates the differentiation of HPCs into $CD4^+$ and $CD8^+$ single positive and $CD4^+CD8^+$ double positive thymocytes. We have previously demonstrated that short-term (3 day) Cellfoam cultures retain multipotency, including the ability to generate myeloid (CFU-GM), erythroid (BFU-E) and T lymphocyte progeny.

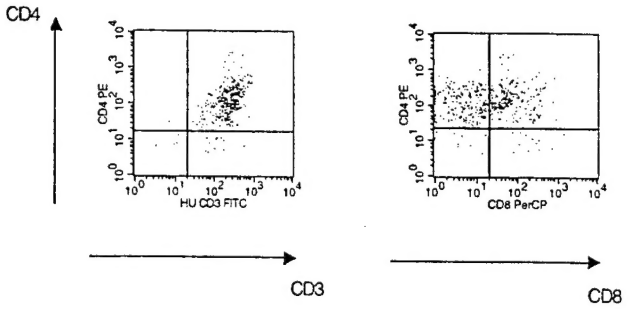
As shown in Table V, when cells from 3 and 6 week Cellfoam and control cultures were harvested and placed in the T lymphopoiesis assay, only cells recovered from Cellfoam generated T cell progeny at both time-points. Cells recovered from the plastic/FN cultures failed to generate T cell progeny. Cells from BMS cultures generated T cell progeny at 3 weeks but not at 6 weeks. Progeny derived from Cellfoam cultures included $CD4^+CD8^+$ double positive thymocytes, as well as $CD4^+$ single positive and $CD8^+$ single positive cells (Figure 16 and Table V). As shown in Figure 16, most of these thymocytes express CD3, an additional indicator of efficient T cell development. To date, no *in vitro* culture system has been shown to efficiently and reproducibly support the maintenance of an HPC population that includes T cell progenitors. As the assessment of multipotency in most assays is typically limited to the generation of myeloid and erythroid colonies, the evaluation of T cell progeny greatly enhances our estimation of the true nature of cells cultured long-term in Cellfoam. As the data demonstrate, Cellfoam was able to support T cell progenitor survival to a greater extent than controls. Importantly, Cellfoam provides and effective long-term culture system for the maintenance of multipotent HPCs *ex vivo*. These observations, coupled with the previous data demonstrating efficient

myelopoiesis in the presence of specific cytokines, indicates that Cellfoam offers tremendous opportunities as a production system for a range of white blood cells.

Table V. Evaluation of T cell progenitors from extended cultures

Condition	Time	$CD34^+$ cells	$CD4^+CD8^+$	$CD4^+$	$CD8^+$
plastic-FN	3 weeks	5×10^4	0	0	0
BMS	""	""	40%	32%	1%
Cellfoam	""	""	55%	40%	3%
plastic-FN	6 weeks	""	0	0	0
BMS	""	""	0	0	0
Cellfoam	""	""	18%	70%	4%

Figure 17. T-cell phenotype of cells from Cellfoam cultures



Multipotency Conclusion: Cellfoam enables the maintenance of HPC multipotency (as measured by *in vitro* colony-formation and T-lymphopoiesis assays) in cultures not supplemented with cytokines. Over extended periods, the colony-forming ability of HPCs cultured in Cellfoam rises, a finding that correlates with the expansion of HPC numbers as measured by surface antigen phenotyping. In control systems, CFU ability declines over time. The bulk population of cells retains the ability to differentiate along red cell, white cell and lymphoid lineages, demonstrating multipotency of the pool of HPCs.

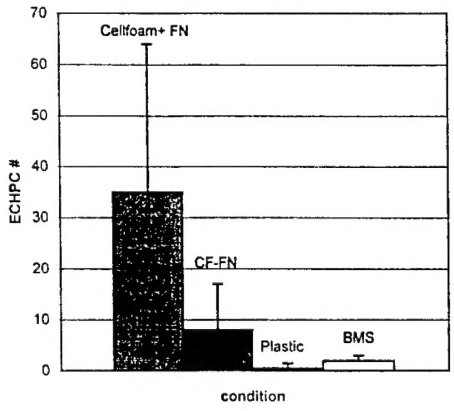
Cellfoam enables enhanced maintenance of ECHPCs: While multipotency assays provide a measure of functionality, they do not specifically address the presence of very long-lived cells. This function, critical to patient engraftment in bone marrow transplantation and likely a cornerstone of sustained *in vitro* stem cell systems, is evaluated by extended culture of HPCs on non-proliferative, irradiated bone marrow stroma layers which enables the analysis of long-lived progenitors termed LTCICs (Long-Term Culture Initiating Cells). The assay is typically conducted by assessing the survival of colony-forming HPCs over several weeks in culture. To evaluate the survival of long-lived progenitors in Cellfoam, a modified LTCIC assay was performed in which HPCs isolated from extended cultures were subsequently grown on bone marrow stroma. As shown in Figure 18a, HPCs cultured in Cellfoam coated with fibronectin maintained ECHPCs over the initial 3 week culture period (9 weeks total in culture). This extended period is beyond the 5-7 week limit of conventional culture systems, and thus extends the classic definition of LTCICs whose survival in LTCIC assays is approximately 6 weeks. To distinguish between traditional LTCICs and these extended survival cells, we refer to the long-lived cells as Extended Culture HPCs or ECHPCs. These studies indicated that Cellfoam cultures yielded 17.5 times as many ECHPCs as bone marrow stroma cultures. Cellfoam units not coated with fibronectin yielded a 4 fold increase in ECHPC activity versus BMS. These data demonstrate that Cellfoam maintains ECHPC activity to a greater extent than conventional controls. As long-term surviving cells are believed to be an important indicator of primitive hematopoietic progenitor content and *in vivo* engraftment capability, these data provide

additional evidence that Cellfoam is advantageous for the culture of HPCs, including primitive progenitors.

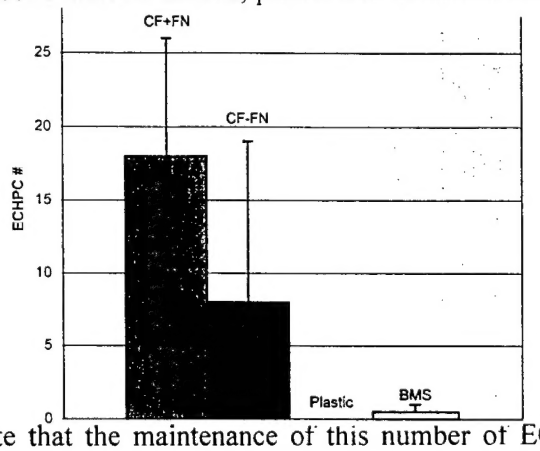
We extended the initial 3 week cultures to cultures performed for 6 weeks followed by traditional LTCIC and CFU assays. In 6 week cultures followed by 6 weeks in LTCIC assays and 2 weeks in colony assays, cells from plastic cultures produced no ECHPCs (Fig. 18). Similarly, BMS cultures had lost virtually all viable ECHPCs. However, Cellfoam cultures yielded encouraging ECHPC numbers. On average, fibronectin-coated Cellfoam units yielded 36 times as many ECHPCs following 6 week cultures as bone marrow stroma cultures, producing 18±8 ECHPCs per 10^4 cells, compared to 0.5±0.7 ECHPCs per 10^4 cells from BMS cultures ($p=0.05$, $n=6$). Fibronectin-coating of Cellfoam units improved ECHPC preservation approximately two-fold over uncoated Cellfoam units. Uncoated Cellfoam units yielded 8±11 ECHPCs per 10^4 cells, a 16 fold increase over bone marrow stroma controls. Compared to the 3 week time-point, 6 week Cellfoam cultures maintained about half as many ECHPCs; this may indicate that static cultures have a somewhat finite ability to maintain long-term culture cells or that selection of more immature, long-lived cells is ongoing. We are currently evaluating how long we can sustain ECHPC activity, and are examining the use of a perfusion approach (see below).

Figure 18. ECHPC studies

a. ECHPC activity in bone marrow stroma, plastic and Cellfoam culture systems after 3 weeks of initial culture.



b. ECHPC activity in bone marrow stroma, plastic and Cellfoam culture systems after 6 weeks of initial culture.



It is important to note that the maintenance of this number of ECHPCs at 11 and 14 weeks represents a significant breakthrough in the cultures of HPCs, as the maintenance of long-lived cells has been reported to correlate with the maintenance of primitive HPCs which includes a subset of cells that may be important contributors to self-renewal and long-term host reconstitution. As our previous data demonstrate, cells cultured over long periods in Cellfoam also retain multipotency, a further indication that the Cellfoam system may represent an enabling

technology for providing the most primitive stem cells required for optimal bone marrow transplantation and repopulation of ablated hosts.

HPC culture in Cellfoam under perfusion conditions: The collective data presented above form the basis of an extended Cellfoam blood cell production system. Recently, work by others has indicated that stem cells may benefit from being placed in culture under perfusion. This concept could be logically incorporated into the Cellfoam system in a straightforward manner by designing perfusion chambers to house the Cellfoam devices. The goal of such an approach would be to impart to stem cells the benefits of the three dimensional Cellfoam environment and perfusion culture. With this in mind, Cytomatrix has designed a continuous perfusion prototype intended to be used in a continuous Cellfoam HPC culture system. This system is a closed, unidirectional series of acrylic chambers which house the Cellfoam units. Multiple chambers are present because it is envisioned that separate compartments for achieving *in vitro* hematopoiesis may be required, as occurs *in vivo*. Mixing chambers with multiple ports are positioned between culture chambers to enable mixing of variable components which will may be used differentially to drive the production of different cells in different areas of the system. As a preliminary step toward integrating Cellfoam with a perfusion system, human HPCs from unfractionated bone marrow samples were seeded in Cellfoam units and placed in a single chamber perfusion prototype. After 2 1/2 weeks, the units were removed, fixed and sectioned. The analysis showed the presence of multiple cells types, both adherent and non-adherent, including hematopoietic progenitors, more differentiated hematopoietic cells, and stromal cells. The development of an advanced perfusion system is envisioned to be pursued in future work to optimize Cellfoam's ability to both preserved/expand and differentiate HSCs in a sustained blood cell production system.

Conclusion: Culture of unfractionated hematopoietic progenitor cells in a perfused Cellfoam system facilitates survival of multiple cell types, including immature hematopoietic cells. This information suggests that development of the Cellfoam perfusion system may enhance the observations presented in this proposal using static Cellfoam systems.

Conclusion:

This report has presented the salient developments of the three dimensional Cellfoam matrix for the long-term preservation, expansion and utilization of primitive multipotent hematopoietic progenitors. In particular, the work presents the findings of Cytomatrix's Phase I DARPA contract which demonstrate that Cellfoam enables the targeted induction of specific blood cell lineages through the use of well defined cytokines. This work is augmented with ongoing work at the company aimed at extending the ability of Cellfoam to preserve and expand primitive stem cells in a cytokine free environment. This work has shown that Cellfoam enables, for the first time, the preservation of stem cells and their numerical and functional expansion in a system that is free of exogenously added cytokines. This capability enables the long-term culture of progenitors that retain their multipotency and longevity. Cytomatrix is moving forward with the enabling Cellfoam technology to develop advanced systems for the preservation, expansion, differentiation and gene therapy of hematopoietic stem cells. As this work proceeds, it will further extend both stem cell preservation and differentiation, and it will explore the integration of Cellfoam devices and the perfusion prototype. The aim of this work will be to provide a consistent, reliable and safe source of high quantities of blood cells for clinical use. With regard to the needs of the military, such blood cells may enable the delivery not only of effective, conventional red blood cells and platelets, but also of novel forms of therapeutic cells such as NK cells, CTLs, other T lymphocytes and white blood cells in general. These innovative cell vehicles may enable the treatment of personnel (and civilians) exposed to novel biological and chemical agents, sources of threat which are becoming increasingly feasible internationally and which pose significant risk to the safety of the military abroad and of the country in general. The development of novel cells using an advanced Cellfoam blood cell production system will be presented to DARPA in the form of a Phase II proposal in December, 1997.